Identification of UV-protective activators of nuclear factor erythroid-derived 2-related factor 2 (Nrf2) by combining a chemical library screen with computer-based virtual screening

Lieder, Franziska ; Reisen, Felix ; Geppert, Tim ; Sollberger, Gabriel ; Beer, Hans-Dietmar ; Auf dem Keller, Ulrich ; Schäfer, Matthias ; Detmar, Michael ; Schneider, Gisbert ; Werner, Sabine

Abstract: Nuclear factor erythroid-derived 2-related factor 2 (Nrf2) is a master regulator of cellular antioxidant defense systems, and activation of this transcription factor is a promising strategy for protection of skin and other organs from environmental insults. To identify efficient Nrf2 activators in keratinocytes, we combined a chemical library screen with computer-based virtual screening. Among 14 novel Nrf2 activators, the most potent compound, a nitrophenyl derivative of 2-chloro-5-nitro-N-phenyl-benzamide, was characterized with regard to its molecular mechanism of action. This compound induced the expression of cytoprotective genes in keratinocytes isolated from wild-type but not from Nrf2-deficient mice. Most importantly, it showed low toxicity and protected primary human keratinocytes from UVB-induced cell death. Therefore, it represents a potential lead compound for the development of drugs for skin protection under stress conditions. Our study demonstrates that chemical library screening combined with advanced computational similarity searching is a powerful strategy for identification of bioactive compounds, and it points toward an innovative therapeutic approach against UVB-induced skin damage.

DOI: https://doi.org/10.1074/jbc.M112.383430

Originally published at:
Lieder, Franziska; Reisen, Felix; Geppert, Tim; Sollberger, Gabriel; Beer, Hans-Dietmar; Auf dem Keller, Ulrich; Schäfer, Matthias; Detmar, Michael; Schneider, Gisbert; Werner, Sabine (2012). Identification of UV-protective activators of nuclear factor erythroid-derived 2-related factor 2 (Nrf2) by combining a chemical library screen with computer-based virtual screening. Journal of Biological Chemistry, 287(39):33001-33013.
DOI: https://doi.org/10.1074/jbc.M112.383430

JBC Papers in Press. Published on July 31, 2012 as Manuscript M112.383430
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.M112.383430

Identification of UV-protective activators of nuclear factor erythroid derived 2-related factor 2 (Nrf2) by combining a chemical library screen with computer-based virtual screening*

Franziska Lieder[1], Felix Reisen[2], Tim Geppert[2], Gabriel Sollberger[3], Hans-Dietmar Beer[3], Ulrich auf dem Keller[1], Matthias Schäfer[1], Michael Detmar[2], Gisbert Schneider[2] and Sabine Werner[1]

[1]From the Institute of Molecular Health Sciences, Department of Biology, ETH Zurich, 8093 Zurich, Switzerland;
[2]Institute of Pharmaceutical Sciences, Department of Chemistry and Applied Biosciences; ETH Zurich, 8093 Zurich, Switzerland;
[3]Department of Dermatology, University Hospital of Zurich, 8006 Zurich, Switzerland

*Running title: *Nrf2 activators in keratinocytes*

To whom correspondence should be addressed: Prof. Dr. Sabine Werner, Institute of Molecular Health Sciences, Department of Biology, Swiss Federal Institute of Technology (ETH) Zurich, Schafmattstrasse 18, 8093 Zurich, Switzerland, Tel: +41 44 633 3941, Fax: +41 44 633 1174, E-mail: sabine.werner@cell.biol.ethz.ch

F.R. and T.G. have equally contributed to this work

Keywords: Nrf2, skin, UV, chemical library, virtual screening

Background: The Nrf2 transcription factor is a master regulator of cellular antioxidant defense systems.
Results: We identified novel Nrf2 activators in keratinocytes with low toxicity and strong UV-protective potential.
Conclusion: Chemical library screening combined with virtual screening is a potent strategy to identify optimized Nrf2 activators.
Significance: Our new Nrf2 activators are potential lead compounds for the development of drugs for skin protection under stress conditions.

SUMMARY

Nuclear factor erythroid derived 2-related factor 2 (Nrf2) is a master regulator of cellular antioxidant defense systems, and activation of this transcription factor is a promising strategy for protection of skin and other organs from environmental insults. To identify efficient Nrf2 activators in keratinocytes we combined a chemical library screen with computer-based virtual screening. Among fourteen novel Nrf2 activators, the most potent compound, a nitrophenyl derivative of 2-chloro-5-nitro-N-phenyl-benzamide, was characterized with regard to its molecular mechanism of action. This compound induced the expression of cytoprotective genes in keratinocytes isolated from wild-type, but not from Nrf2-deficient mice. Most importantly, it showed low toxicity and protected primary human keratinocytes from UVB-induced cell death. Therefore, it represents a potential lead compound for the development of drugs for skin protection under stress conditions. Our study demonstrates that chemical library screening combined with advanced computational similarity searching is a powerful strategy for identification of bioactive compounds, and points toward an innovative therapeutic approach against UVB-induced skin damage.

INTRODUCTION

Ultraviolet (UV) irradiation, gamma irradiation or various toxic or irritant chemicals challenge the skin, the outermost surface of our body. Many of these insults cause oxidative stress through enhanced production of reactive oxygen species (ROS). This results in cell damage and inflammation, and also contributes to skin aging and even neoplastic transformation (1, 2). One of the most important regulators in the defense against oxidative stress is the transcription factor

nuclear factor erythroid derived 2-related factor 2 (Nrf2). It is ubiquitously expressed, and particularly high expression levels are seen in epithelial cells, including keratinocytes of the skin (3). Nrf2 belongs to the Cap'n'Collar family of transcription factors, which also includes the related Nrf1 and Nrf3 proteins, as well as p45NF-E2, Bach1 and Bach2 (4, 5). Under basal conditions Nrf2 is predominantly present in the cytoplasm where it is anchored to the actin-binding protein Keap1 that mediates its degradation through the ubiquitin-proteasome pathway. Electrophilic substances can activate Nrf2 through modification of Keap1 cysteine residues (6). This results in the stabilization of Nrf2 and its accumulation in the nucleus (7). In addition, it has been suggested that ROS can activate certain kinases, which in turn phosphorylate Nrf2, resulting in its stabilization and activation (8). However, we could not verify the latter mechanism in keratinocytes, and it may therefore be cell-type specific (9). Upon nuclear translocation, Nrf2 dimerizes with small Maf proteins or other leucine zipper proteins and binds as a heterodimer to *cis*-acting elements in the promoters of its target genes, designated as antioxidant response elements (AREs). The core consensus sequence of an ARE was characterized by deletion analysis and is defined as 5'-TGACnnnGC-3' (10).

Genes that are regulated by Nrf2 encode, among others, proteins that help to control the cellular redox state and protect the cell against oxidative damage or toxic chemicals. These proteins include several ROS detoxifying enzymes and other antioxidant proteins, including NAD(P)H dehydrogenase quinone 1 (NQO1), different glutathione S-transferases (GST), the regulatory and catalytic subunits of the glutathione biosynthesis enzyme γ-glutamylcysteine ligase (γ-GCL; GCLM and GCLC), peroxiredoxins (PRDX) 1 and 6, and heme oxygenase-1 (HO-1). Studies with knockout mice highlighted the important role of Nrf2 in the cellular stress response. Nrf2-deficient animals are more susceptible to diseases related to oxidative stress compared to their wild-type littermates (11). In the skin, loss of Nrf2 prolonged the inflammatory response after wounding (3). Most importantly, incidence and multiplicity of chemically-induced skin tumors were strongly enhanced in transgenic mice expressing a dominant-negative mutant of Nrf2 in keratinocytes (12) as well as in Nrf2 knockout mice (13). Since the basal activity was shown to mediate these protective effects (12), it was suggested that further activation of Nrf2 could be used for cancer prevention. Indeed, several preclinical and clinical studies with Nrf2-activating compounds support this hypothesis (14, 15). Furthermore, transgenic mice expressing a constitutively active (ca)Nrf2 mutant in keratinocytes were protected from UVB-induced apoptosis in the back skin epidermis. Expression of caNrf2 resulted in the activation of Nrf2 target genes and, therefore, reduced the levels of intracellular ROS in keratinocytes (16).

Due to these cytoprotective functions of Nrf2 *in vivo*, it is of major interest to identify Nrf2-activating compounds in keratinocytes that can be exploited therapeutically to impede skin-damaging effects, e.g. of UV irradiation. One of the best-characterized Nrf2 activators is the broccoli sprout component sulforaphane (SFN), which activates Nrf2 by chemical modification of highly reactive cysteine residues of Keap1 (17). Topical application of broccoli sprout extracts on SKH-1 hairless mice strongly reduced the tumor multiplicity and total tumor burden in a UV-induced skin carcinogenesis study (18). However, sulforaphane exerts Nrf2-independent effects, e.g. through direct regulation of glutathione levels (19, 20), and we recently showed that long-term treatment of the skin with this compound causes an ichthyosis-like skin phenotype (21). In addition, a common feature of many cosmetic ingredients that cause skin sensitization is their capability to activate Nrf2 as shown in reporter gene assays with cultured cells (22). Therefore, it is desirable to identify and characterize novel Nrf2-activating compounds with high specificity and activity but low toxicity, which can be used for *in vivo* application. In this study we used a novel strategy to identify Nrf2 activating compounds - combining a chemical library screen with computer-based virtual screening. We identified novel and highly potent Nrf2-activating compounds in keratinocytes with a remarkable cytoprotective function.

EXPERIMENTAL PROCEDURES

Chemicals – Rosiglitazone, T0070907, compound **1** (GW9662) and BADGE were from Enzo Life Sciences (Lausen, Switzerland). Compounds

identified by the virtual screen were obtained from Asinex (Rijswjk, The Netherlands), InterBioScreen (Moscow, Russia) or Specs (Wakefield, RI). Recombinant human Keap1 protein was purchased from Origene (Rockville, MD). Forskolin was purchased from Sigma (Munich, Germany) and human tumor necrosis factor-alpha (TNF-α) was from PeproTech (Hamburg, Germany).

Cell culture – Cells were cultured at 37 °C in a humid atmosphere containing 5 % CO_2 (v/v). The immortalized, non-tumorigenic human HaCaT keratinocyte cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10 % fetal calf serum (FCS; Invitrogen, Paisley, UK) and 1 % penicillin/streptomycin.

Spontaneously immortalized keratinocytes from Nrf2 knockout (23) mice and their wild-type littermates were obtained in our laboratory by serial passaging of primary cells. The latter were obtained from 3-day old wild-type and Nrf2 knockout mice (3). While most cells became senescent upon multiple passaging, a few foci of immortalized cells appeared, which were further passaged. Cells were maintained in defined keratinocyte serum-free medium (dk-SFM) (Invitrogen) supplemented with 10 ng/ml epidermal growth factor, 10^{-10} M cholera toxin and 1 % penicillin/streptomycin (all from Sigma).

Primary human foreskin keratinocytes (HFK) were established from human foreskin obtained from the Department of Dermatology, University Hospital Zurich. They were cultured up to six passages in Keratinocyte-SFM supplied with epidermal growth factor and bovine pituitary extract (Invitrogen).

Construction of ARE luciferase reporter plasmids – ARE luciferase reporter plasmids were generated using the pGL3-promoter vector (Promega, Madison, WI), which includes an SV40 promoter upstream of the firefly luciferase gene. Double stranded oligonucleotides were synthesized containing a part of the promoter of the respective Nrf2 target gene, including the ARE (underlined). The different inserts are 1xARE-mGclm (forward strand 5'-**C**CTGGAAGACAAT GACTAAGCAGAAA**C**-3' and reverse strand 5'-**TCGAG**TTTCTGCTTAGTCATTGTCTTCCAG **GAGCT**-3'), 1xARE-ratNqo1 (forward strand 5'-**C**TCTAGAGTCACAGTGACTTGGCAAAATCT GA**C**-3' and reverse strand **TCGAG**TCAGATTT TGCCAAGTCACTGTGACTCTAGA**GAGCT**-3') and 8xARE-mGclm (forward strand 5'-**C**ATGACTAAGCACCCATGACTAAGCACCC ATGACTAAGCACCCATGACTAAGCACCCA TGACTAAGCACCCATGACTAAGCACCCAT GACTAAGCACCCATGACTAAGCACCC**C**-3' and reverse strand 5'-**TCGAG**GGGTGCTTAGT CATGGGTGCTTAGTCATGGGTGCTTAGTCA TGGGTGCTTAGTCATGGGTGCTTAGTCATG GGTGCTTAGTCATGGGTGCTTAGTCATGGG TGCTTAGTCAT**GAGCT**-3'). The sequence was extended (bold) to generate 5'-terminal *Sac*I and 3'-terminal *Xho*I restriction sites. After annealing of the complementary oligonucleotides, the double stranded oligonucleotides were inserted into the pGL3 vector upstream of the SV40 promoter. In addition, a plasmid was generated with eight copies of the minimal functional ARE sequence present in the murine *Gclm* (ATGACTAAGCA) gene promoter. Each ARE copy was linked with the sequence 5'-CCC-3' and 5'-GGG-3' on the opposite strand to another copy. Oligonucleotide synthesis and sequencing of the final plasmids were performed by Microsynth (Balgach, Switzerland). Additional reporter plasmids with a nuclear factor-kappa B (NF-κB) binding site (pNF-κB-luc), a serum response element (SRE; pSRE-luc) or a cAMP response element (CRE; pCRE-luc) were obtained from Clontech (Mountain View, CA).

Transient transfection and luciferase reporter assays – Transfection of HaCaT cells and immortalized mouse keratinocytes was performed with linear polyethyleneimine (Polysciences, Warrington, PA). Cells were plated at a density of $2x10^5$ cells/well in 12-well plates (Nunc, Roskilde, Denmark) and grown to 80 % confluency (24 h). After two washing steps with PBS, 1 ml Opti-MEM I (Invitrogen) was added and the cells were co-transfected with 2 µg of the ARE luciferase reporter plasmid and 0.01 µg of the phRL-CMV *Renilla* luciferase vector plasmid (Promega) as an internal control for transfection efficiency when different reporter plasmids were compared. 3.8 µl of polyethyleneimine solution (1 mg/ml) was then added, the mixture was vortexed, incubated at room temperature for 15 min to allow complex formation and added to the cells. After 24 h the cells were washed with PBS and incubated with fresh culture medium containing tBHQ (Merck, Darmstadt, Germany), sulforaphane (Sigma) or

DMSO as negative control. Cells were harvested 24 h later using *Passive Lysis Buffer* (Promega). Firefly and *Renilla* luciferase activities were measured in the lysates using the Dual-Luciferase Reporter Assay System kit (Promega) according to the manufacturer's instructions. Luciferase activity was determined in a MicroLumatPlus LB96V luminometer (Berthold Technologies, Bad Wildbad, Germany). For the library screen and the validation of the screening results we seeded $9x10^6$ cells into 15 cm dishes and added a transfection mixture containing 90 µg pGL3-8xARE-mGclm reporter plasmid, 171 µl polyethyleneimine and 4.2 ml 0.9 % (w/v) NaCl. Since all cells were transfected in the same plate with the same reporter plasmid, co-transfection with a *Renilla* luciferase plasmid was not performed in this case. Luciferase activity was measured by adding luciferase assay reagent consisting of 20 mM tricine, 2.67 mM $MgSO_4$, 0.1 mM ethylenediaminetetraacetic acid, 33.3 mM dithiothreitol, 0.27 mM coenzyme A, 0.53 mM adenosine triphosphate (all from Sigma) and 0.47 µM luciferin potassium salt (Synchem, Kassel, Germany), pH 7.8.

Screening of a chemical library – The LOPAC library (Sigma), containing 1280 pharmacologically active compounds, was used for the screen. All chemicals of this library are arranged in 96-well plates, dissolved in DMSO, and have a concentration of 10 mM. To generate 1 mM stock plates aliquots of the compounds were diluted with DMSO in fresh 96-well tissue culture plates. HaCaT cells were transiently transfected with the reporter vector pGL3-8xARE-mGclm (see above). Cells were trypsinized 24 h after transfection and seeded into 96-well plates (25,000 cells/well). 24 h later the medium was replaced by fresh growth medium and the cells were treated for 24 h with 10 µM of the chemicals of the library. Afterwards cells were washed with PBS, lysed in *Passive Lysis Buffer* (Promega), and luciferase activity was measured as described above using the luciferase assay reagent.

Screening compound library – For virtual screening we compiled a collection of 1,033,267 compounds from selected commercial vendors: Specs Natural Products v08/2010, Specs Screening Collection v08/2010 (Specs, Delft, The Netherlands); Interbioscreen Natural Compound Library v08/2010, Interbioscreen Synthetic Compound Collection v08/2010 (Interbioscreen, Moscow, Russia); Asinex Gold v08/2010, Asinex Platinum v08/2010, Asinex Synergy v08/2010 (Asinex, Moscow, Russia). All compounds were pre-processed using the 'wash' function (i.e., deprotonation of strong acids, protonation of strong bases) in the Molecular Operating Environment (MOE) software (The Chemical Computing Group Inc., Montreal, Canada). For each compound a single heuristic three-dimensional conformation was generated with CORINA 3.46 (Molecular Networks, Erlangen, Germany) for autocorrelation (LIQUID) and graph (PoLiMorph) descriptor calculation. For ligand-based virtual screening, we computed similarity values between queries and all screening pool compounds. We considered pool compounds as virtual hits if they were retrieved among the top 1 % most similar compounds for both descriptors and among the top 100 compounds using the added rank of both methods for sorting. From the resulting virtual hit lists, we manually selected compounds for bioactivity determination.

Quantitative Real-time RT-PCR (qRT-PCR) – RNA was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer. cDNA was generated using the iScript™cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Relative gene expression was determined using the Roche LightCycler 480 SYBR Green system (Roche, Rotkreuz, Switzerland). The following set of primers were used: mGclc-for 5'-AACAAGAAA CATCCGGCATC-3' and mGclc-rev 5'-CGTAGC CTCGGTAAAATGGA-3', mGclm-for 5'-TCCCATGCAGTGGAGAAGAT-3' and mGclm-rev 5'-AGCTGTGCAACTCCAAGGAC-3', mNqo1-for 5'-CTGGCCCATTCAGAGAAGAC-3' and mNqo1-rev 5'-GTCTGCAGCTTCCAGCT TCT-3', mRps29-for 5'-GGTCACCAGCAGCTC TACTG-3' and mRps29-rev 5'-GTCCAACTTAA TGAAGCCTATGTCC-3', hGCLC-for 5'-GGAA GGAAGGTGTGTTTCCTGG-3' and hGCLC-rev 5'-ACTCCCTCATCCATCTGGCAA-3',hGCLM-for 5'-CCAGATGTCTTGGAATGCACTG-3' and hGCLM-rev 5'-AGGACTGAACAGGCCAT GTCA-3', hNQO1-for 5'-GTGATATTCCAGTT CCCCCTGC-3' and hNQO1-rev 5'-AAGCACTG CCTTCTTACTCCGG-3', and hRPL27-for 5'-TCACCTAATGCCCACAAGGTA-3' and hRLP27-rev 5'-CCA CTTGTTCTTGCCTGTC

TT-3'. Each sample was analyzed in duplicates and the amplification of cDNA of the housekeeping genes *mRps29* (murine) or *hRPL27* (human) was used for normalization.

MTT and LDH assays – Human foreskin keratinocytes (HFK) were seeded in 24-well plates (45,000 cells/well) and cultured overnight. Cells were treated for 24 h with different concentrations of test compounds (1 µM - 100 µM). For the MTT assay the medium was supplemented with 100 µl of MTT solution (5 mg/ml in sterile PBS) (Sigma). After 2 h incubation, the supernatant was aspirated and the cells were lysed in 200 µl 40 mM HCl/isopropanol for 10 min at RT. The reaction was stopped by addition of an equal volume of water. The optical density was measured at 590 nm. To measure LDH release the CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega) was used according to the manufacturer's protocol.

Mass spectrometry analysis of cysteine modification of Keap1 – Recombinant human Keap1 protein (6 pmol) was incubated with a five-fold molar excess of test compound in a total volume of 100 µl in 50 mM Tris-HCl, pH8, 150 mM NaCl for 3 h at room temperature. Subsequently, the protein was digested by addition of 10 ng of mass spectrometry grade trypsin (Promega) and incubated for 16 h at 37 °C. Peptides were cleaned up using µC18-ZipTip columns (Millipore) and analyzed on an LTQ-Orbitrap XL ETD mass spectrometer (Thermo Fischer Scientific, Bremen, Germany) coupled to an Eksigent-Nano-HPLC system (Eksigent Technologies, Dublin (CA), USA). Solvent composition at the two channels was 0.2 % formic acid, 1 % acetonitrile for channel A and 0.2 % formic acid, 80 % acetonitrile for channel B. Peptides were loaded on a self-made tip column (75 µm x 80 mm) packed with reverse phase C18 material (AQ, 3 µm, 200 Å, Bischoff GmbH, Leonberg, Germany) and eluted with a flow rate of 200 nl per min by a gradient from 0 to 10 % of B in 5 min, 47 % B in 55 min, 97 % B in 58 min. Full-scan MS spectra (300-2000 m/z) were acquired with a resolution of 60,000 at 400 m/z after accumulation to a target value of 500,000. Collision induced dissociation (CID) MS/MS spectra were recorded in data dependent manner in the ion trap from the three most intense signals above a threshold of 500, using a normalized collision energy of 35 % and an activation time of 30 ms. Charge state screening was enabled and singly charge states were rejected. Precursor masses already selected for MS/MS were excluded for further selection for 90 s and the exclusion window was set to 20 ppm. The size of the exclusion list was set to a maximum of 500 entries. Peak lists were extracted from raw data files using Mascot Distiller (Matrix Science) and searched against a UniProtKB/Swiss-Prot database release 15.13 using the Mascot v2.3 search engine with the following parameters: Trypsin for enzyme specificity allowing up to one missed cleavage; oxidation (M, C), di- and trioxidation (C) and mass shifts at C corresponding to the test compound as variable modifications; parent mass error at 10 ppm, fragment mass error at 0.8 Da.

Analysis of intracellular ROS levels – HFKs were seeded in 12-well plates and grown overnight. Medium was changed and cells were incubated with 100 µM 2'7'-dichloro-dihydrofluorescein diacetate (H_2DCFDA) (Invitrogen) for 30 min in the CO_2 incubator. The cells were washed once with HEPES buffered salt solution (HBSS; 25 mM HEPES, 120 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 25 mM $NaHCO_3$, 15 mM glucose, pH 7.4), and subsequently incubated in fresh medium with different concentrations of test compounds (1 µM - 100 µM). Fluorescence was measured after 0 - 24 h using a fluorescence multiwell plate reader (Spectramax M2, Molecular Devices, Ismaning, Germany) with excitation and emission wavelengths of 485 nm and 530 nm.

UVB irradiation – HFKs were seeded in 12-well plates (90,000 cells/well) and grown overnight. After treating the cells for 1 h, 4 h or 24 h with 5 µM SFN, 50 µM compound **1**, 50 µM compound **1a**, or DMSO as control the medium was replaced by fresh growth medium. Cells were then irradiated with 50 mJ/cm^2 UVB using a Medisun FH-54 lamp (Schulze & Böhm, Huerth, Germany) equipped with six UVB-TL/12 bulbs (9 W each; Philipps, Amsterdam, Netherlands), which emit UVB light in the range of 280 to 315 nm with a peak emission at 312 to 315 nm. 24 h later MTT or LDH assays were performed.

Statistical analysis – Statistical analysis was performed using the PRISM software (Graph Pad Software Inc., La Jolla, CA). A two-way ANOVA analysis with Bonferroni post-test to compare multiple groups was performed. *$P \leq 0.05$,

$^{**}P \leq 0.005$, $^{***}P \leq 0.001$.

RESULTS

Screening of a chemical library for new Nrf2 activators – To identify novel Nrf2 activators in keratinocytes we performed a chemical library screen. For this purpose we first tested the efficiency of several AREs (Supplementary Table S1) to activate a luciferase reporter gene with a minimal promoter in the human HaCaT keratinocyte cell line. The ARE from the murine (m) *Gclm* gene was identified as particularly potent (data not shown) and was therefore chosen for further studies. Due to the high homology between murine and human Nrf2 (5), most mouse AREs, including the *mGclm* ARE, can be used to study Nrf2 activity in human cells. First, we generated a multimer with eight copies of the ARE (pGL3-8xARE-mGclm), which further enhanced the efficiency in the activation of a luciferase reporter gene compared to the monomeric ARE (Supplementary Fig.S1). This reporter gene construct was subsequently used to screen the *Library of Pharmacologically Active Compounds* (LOPAC$^{1280\ TM}$, Sigma-Aldrich), which comprises 1280 bioactive, well-characterized compounds that act via 56 classes of pharmacological targets. HaCaT keratinocytes were transiently transfected with pGL3-8xARE-mGclm and treated for 24 h with the compounds from the library (assay concentration 10 µM each). The solvent DMSO was used as a negative control, and the known Nrf2 activators *tert*-butylhydrochinone (tBHQ) and SFN served as positive controls. Three independent screening rounds were conducted. A two-fold increase in luciferase activity was set as threshold for a positive hit. Forty-five hit compounds were discovered in at least one screening experiment. Six of them were positive in each screen, and seven were positive in two screens. These 13 putative Nrf2-activating substances were chosen for further analysis (compounds **1**-**13**; Table 1). The 32 hit compounds appearing in only one of the three screening runs (Supplementary Table S2) were not further investigated.

Three of the 13 hit compounds had been previously described as activators of Nrf2. These included iodoacetamide (24), parthenolide (25), and quercetin dehydrate (26). In addition, 3,4-dichloroisocoumarin is structurally related to the known Nrf2 activator coumarin (27), and tBHQ is a derivative of the aromatic compound hydroquinone (28), which we identified in our screen. The identification of these known Nrf2 activators corroborates the efficiency and suitability of the approach.

Validation of the screening results using HaCaT keratinocytes – To validate the eight novel potential Nrf2 activators (hit compounds) we extended the reporter assays with HaCaT cells using different incubation times (5 h, 10 h or 24 h) and increasing concentrations of the compounds (0.1 µM, 10 µM, 25 µM and 50 µM). Furthermore, we tested the hit compounds on cells transfected with the pGL3-promoter vector (without ARE) to determine if the activation is indeed ARE-dependent. None of the eight compounds activated the reporter after transfection with this control vector, neither after 5 h or 10 h (data not shown) nor after 24 h (Fig.1A). By contrast, luciferase activity was strongly enhanced in cells transfected with the pGL3-8xARE-mGclm vector within 10 h (results not shown) and 24 h after addition of the compounds (Fig.1B), and the activation occurred in a dose-dependent manner. The strongest induction (5.5-fold) was achieved with 2-chloro-5-nitro-N-phenyl-benzamide (compound **1**), followed by arecaidine propargyl ester hydrobromide (compound **2**) and 4-phenyl-3-furoxan-carbonitrile (compound **3**). (Z)-gugglesterone (compound **4**), Bay 11-7085 (compound **5**), SKF 83959 hydrobromide (compound **6**), spiperone hydrochloride (compound **7**) and N-p-tosyl-L-phenylalanine chloromethyl ketone (compound **8**) caused a 2 to 3.5-fold increase in luciferase activity. For compounds **3**, **4**, **5** and **8** a dramatic reduction of reporter gene expression was observed with increasing concentrations, most likely reflecting cytotoxicity. Therefore, we used the concentration that induced the maximal activation for all further experiments.

To determine if the hit compounds also activate gene expression via other AREs, we performed reporter assays with HaCaT cells transfected with the pGL3-1xARE-rNqo1 plasmid that contains the ARE and some additional flanking sequences from the promoter region of the rat *Nqo1* gene. Indeed, all hit compounds also activated gene expression via this ARE in a dose-dependent manner (Supplementary Fig.S2), and

compound **1** was again identified as the most potent inducer. Therefore, it seems likely that the compounds mediate gene expression via AREs and not via gene-specific flanking sequences.

The hit compounds induce the expression of established Nrf2 target genes in HaCaT cells – We next determined if the hit compounds induce the expression of the known Nrf2 target genes *GCLC*, *GCLM* and *NQO1* (29). For this purpose HaCaT cells were treated for 8 h, 24 h or 48 h with the hit compounds or with tBHQ or SFN as positive controls (Fig.2). Using quantitative Real-Time RT-PCR (qRT-PCR) we observed an increase in the mRNA levels of *GCLC, GCLM* and *NQO1* with most of the hit compounds. The time course of induction was target gene dependent. Compounds **1**, **2**, **3**, **4** and **5** and to a lesser extent compound **8** induced the expression of all target genes, whereas only two of these genes were induced by compounds **6** and **7**, and their effect was very mild. Taken together, six of the eight hit compounds efficiently induced the expression of Nrf2 target genes in HaCaT cells.

The hit compounds act via Nrf2 – To determine if the activation of the previously tested cytoprotective genes by the hit compounds requires Nrf2, we tested their regulation in spontaneously immortalized keratinocytes isolated from Nrf2 knockout mice and their wild-type littermates. Lack of Nrf2 expression in cells from the knockout mice was verified by qRT-PCR (data not shown).

Compounds **1**, **2**, **4** and **8** indeed induced the expression of *Gclc*, *Gclm* and *Nqo1* in cells from wild-type mice, but not in cells from Nrf2 knockout mice (Fig.3A-C). The only exception was compound **3**, which also induced a mild upregulation of *Gclm* expression, indicating that this gene can also be regulated by other transcription factors in response to compound **3**. Surprisingly, compound **5** was not able to induce the expression of Nrf2 target genes in murine keratinocytes. Similar to the results obtained with HaCaT cells, compounds **6** and **7** did not or only mildly activate Nrf2 target gene expression in murine keratinocytes. Therefore, these compounds were not further characterized.

Identification of additional Nrf2 activators by virtual screening based on the hit compounds – To identify additional and possibly improved Nrf2 activators and to determine structural features that are important for Nrf2 activation, we selected six of the hit compounds (**1-5**, **8**) as queries for ligand-based virtual screening experiments (similarity searching) using the computational methods LIQUID (30) and PoLiMorph (31). We compiled a database comprising 1,033,267 commercially available compounds, which was screened using a consensus scoring scheme as described previously (32). We selected in total 20 compounds for *in vitro* testing (Table 2) from the six resulting hit lists. At least two compounds were chosen for each query. Their capability to activate Nrf2 in HaCaT cells using reporter gene assays with the pGL3-8xARE-mGclm construct was tested in three different concentrations (1 μM, 10 μM, 100 μM). Six out of 20 test compounds (Table 2, highlighted in grey) increased reporter gene activity in three independent experiments by more than 1.5-fold. These included three analogues of compound **1** (**1a-1c**), a bioisoster of compound **2** (**2a**), and two analogues of compound **5** (**5a** and **5b**) (Supplementary Table S3).

Subsequently, we tested the ability of these six new compounds to induce the expression of the Nrf2 target gene *Gclc* in murine keratinocytes from wild-type mice (Fig.4A). Compounds **1c**, **2a** and **5b** did not upregulate *Gclc* on the mRNA level (data not shown). However, compound **1b** and in particular compound **1a** caused a stronger increase in the expression of *Gclc* on the mRNA level compared to the original compound **1**. Furthermore, treatment with compound **5a** resulted in a higher upregulation of this Nrf2 target gene compared to the hit compound **5**, although the overall activity of both compounds was low compared to the other, more active compounds. None of these compounds enhanced the expression of *Gclc* in keratinocytes lacking Nrf2.

For the further characterization of the most interesting hit compounds (**1**, **2**, **5**) and their analogues (**1a**, **1b**, **5a**), in particular with regard to their potential future *in vivo* application, we used primary human foreskin keratinocytes (HFK). In general, the increase in the mRNA levels of the target gene *GCLC* was lower than in immortalized murine keratinocytes. Nevertheless, compounds **1**, **1a** and **1b** as well as **2** enhanced the expression of this gene in HFKs. Remarkably, compounds **1**, **1a** and **1b** were even more potent than SFN in this assay. Since compounds **5** and **5a** had no or only a

minor effect on *GCLC* expression (Fig.4B), we next focused our analysis on compounds **1** and **1a**.

*Compounds **1** and **1a** specifically activate ARE reporter genes.* We first determined the specificity of compounds **1** and **1a** with regard to reporter gene activation. For this purpose we tested if these compounds also activate reporter genes with response elements for unrelated transcription factors (NF-κB binding site, serum response element or cAMP response element). While these reporter genes were efficiently activated by TNF-α, serum or forskolin, respectively, compounds **1, 1a,** sulforaphane and tBHQ did not activate these reporter genes at different concentrations (Supplementary Fig.S3). This result demonstrates the specificity of these compounds for AREs.

*Compounds **1** and **1a** activate Nrf2 in a PPARγ independent manner* – Compound **1** (2-chloro-5-nitro-N-phenyl-benzamide; GW9662) is a potent (IC50 in the nanomolar range) and irreversible antagonist of the peroxisome proliferator-activated receptor gamma (PPARγ) (33), a nuclear receptor transcription factor. PPARγ is expressed at low levels in human keratinocytes (34), which we confirmed for HFKs using qRT-PCR (data not shown). We next tested if modulation of PPARγ activity affects the activity of Nrf2. For this purpose we tested the effect of T0070907 and bisphenol A diglycidyl ether (BADGE), two other PPARγ antagonists (35, 36), and of the PPARγ activator rosiglitazone (37), on *GCLC* and *NQO1* gene expression in keratinocytes. Treatment with BADGE or rosiglitazone did not affect the expression of these Nrf2 target genes, whereas T0070909 treatment increased their expression (Fig.5). Compound **1** and T0070907 are structurally related, whereas BADGE has a different structure (Supplementary Fig.S4). Since compound **1** and T0070907 bind covalently to cysteine residues of PPARγ (Cys285 or Cys313, respectively) (33, 35), it seems likely that they activate Nrf2 through modification of cysteine residues in Keap1 rather than through inhibition of PPARγ activity. To test this hypothesis, we first treated recombinant human Keap1 with iodoacetamide under reducing conditions to identify the cysteines, which are accessible for covalent modification. Ten alkylated cysteine residues, including several which are modifiable by Nrf2 activating compounds (38), were modified under these conditions (data not shown). We then incubated recombinant full-length human Keap1 protein with compound **1a** and subsequently analyzed tryptic digests by mass spectrometry for cysteine containing peptides that had been covalently modified by the test compound. Indeed, we observed a stable modification by compound **1a** of cysteine residue 368 (Supplementary Fig.S5), a known highly reactive sensor cysteine in Keap1 (39), which could also be modified by iodoacetamide (see above). These findings strongly suggest that compound **1a** activates Nrf2 via Keap1, but not via PPARγ.

Compounds 1 and 1a reveal only low toxicity for primary human keratinocytes – We next analyzed the toxicity of compounds **1** and **1a** for primary human keratinocytes. Remarkably, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays revealed a significantly lower toxicity of these compounds at higher concentrations compared to SFN and tBHQ (Fig.6A). This was confirmed by analysis of lactate dehydrogenase (LDH) activity in the cell supernatant (Fig.6B). The presence of this cytosolic enzyme in the medium reflects cell lysis. The discrepancy in the result obtained with SFN in the MTT and LDH assays may result from an interference of SFN with the LDH activity assay as suggested by the increase in the levels of β-actin in the cell supernatant at high concentrations of SFN (Supplementary Fig.S6A). To determine the effect of compounds **1** and **1a** on ROS production, keratinocytes were treated with 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) and subsequently analyzed for DCF fluorescence. Only a moderate ROS production was seen with both compounds, which was comparable to the production induced by SFN (Fig.6C). By contrast, ROS production was much higher in tBHQ-treated cells. The functionality of the assay was confirmed by analysis of cells treated with glucose oxidase (GO), which results in continuous production of hydrogen peroxide (Supplementary Fig.S6B).

Compounds 1 and 1a protect keratinocytes from UVB-induced apoptosis - Since SFN protects keratinocytes *in vitro* and in *vivo* against UVB damage (18, 40), we tested if compounds **1** and **1a** have a similar photoprotective potential. For this purpose, HFK were treated with Nrf2 activators

for different time periods (1 h, 4 h or 20 h) prior to irradiation with a physiological dose of UVB (50 mJ/cm^2). Remarkably, already a one-hour incubation with compounds **1** or **1a** or with SFN reduced the LDH release compared to DMSO-treated cells. The reduction was even stronger after a 20 h pre-treament, and cells treated with compounds **1** or **1a** were more protected compared to SFN-treated cells (Fig.7A). The UVB-protective effect of both compounds was verified by MTT assay, where pre-treatment with these compounds enhanced the viability of UVB-irradiated keratinocytes (Fig.7B). These findings reveal that the novel Nrf2 activators identified in this study have a potent cytoprotective potential.

DISCUSSION

Activation of the cytoprotective transcription factor Nrf2 is a promising strategy for protection of cells under stress conditions. Nrf2 activation can be achieved by various low molecular weight compounds, of which some are in clinical trials for cancer prevention (41). Recently, novel Nrf2 activators have been identified by screening of chemical libraries in cell culture assays (42-44). Of particular interest is a screen that used the LOPAC library (published by C. Klaassen in PubChem BioAssay, ID 624149). Surprisingly, there was only one overlap between this screen and our library screen – hydroquinone, a previously identified Nrf2 activator (see Results). By contrast, the new compounds that we identified are not included in the positive hits of this screen. These differences may result from the use of different cell lines (transformed breast cancer cell line versus non-transformed keratinocyte cell line). Importantly, none of the previously performed chemical library screens had been combined with a virtual screening approach. The latter represents an efficient strategy to identify novel compounds exhibiting a desired biochemical activity (45). In addition, UV-protective functions of these compounds have not been demonstrated. The possibility of using Nrf2 activators for cell protection under stress conditions is particularly promising for the skin, as demonstrated by the beneficial effect of sulforaphane for the treatment of skin blistering or protection from UV damage (40, 46, 47). However, sulforaphane has various Nrf2-independent effects (19, 20) and also induces pathological abnormalities in the skin upon long-term application (21). Therefore, identification of novel and specific Nrf2 activators with low toxicity is highly desirable. Here we used a combination of chemical library screening and virtual screening to identify improved Nrf2 activators in keratinocytes, which revealed low toxicity for keratinocytes combined with a potent cytoprotective potential.

The chemical library screen identified six novel Nrf2 activators in keratinocytes. Compound **4** ((Z)-gugglesterone) is a plant sterol for which multiple targets have been described, including several nuclear hormone receptors as well as IκB kinase (48, 49). Due to this broad activity spectrum, we decided not to further characterize this compound with regard to Nrf2 activation, in particular since none of the analogues revealed Nrf2 activating capacity. This was also the case for compound **3** (4-phenyl-3-furoxan-carbonitrile), which releases nitric oxide (NO) under the action of thiol co-factors, such as cysteines (50). NO was shown to cause (*S*)-nitrosation of cysteine residues in Keap1 and thereby activate Nrf2 (51, 52). Due to the cytotoxicity of NO, which was also reflected in the experiments with compound **3**, the latter was also not further investigated. Obvious toxicity at higher concentrations was also observed for compound **8** (tosyl-L-phenyl-alanine chloro-methyl ketone), and none of its analogues was able to activate Nrf2. Therefore, we decided to focus on the remaining three compounds. Among them, compound **1** and its derivatives appeared most interesting for the following reasons: (i) Compound **1** caused the strongest activation of the ARE reporter genes and of the endogenous Nrf2 target genes; (ii) a class of structural analogues are functionally equivalent with regard to Nrf2 activation, (iii) neither compound **1** nor its derivatives revealed obvious toxicity in the reporter assays and (iv) they are structurally unrelated to previously published Nrf2 activators. The difference between compounds **1** and **1a** is a chinoline versus a phenyl group. *Para*-substituted nitrobenzene derivatives of **1** were active, while *meta*-substituents were not tolerated. Similarly, derivatives **5a** and **5b** indicate a critical region in the scaffold. This points to preliminary structure-activity relationships that could be explored in future structure-based hit-to-lead optimization. The successful scaffold-hop (53) from compound **2** to compound **2a** is the most surprising finding

among the newly found actives. The quinuclidine **2a** features the tertiary nitrogen and a potential hydrogen-bond acceptor of the template **2**, but offers alternative scaffold architecture. This example represents a pair of isofunctional chemotypes, with **2a** being a second promising candidate, in addition to compound **1a**, for further chemical exploration.

The published target of compound **1** is PPARγ (33), which is irreversibly inhibited. However, it seems unlikely that inhibition of PPARγ is important for the effect on Nrf2, since a structurally unrelated PPARγ inhibitor did not activate Nrf2 target genes and since PPARγ activation also had no effect. Rather, it seems likely that compound **1** and its derivatives act via Keap1 due to the presence of reactive cysteines. Consistent with this hypothesis, mass spectrometry analysis of PPARγ modified by compound **1a** had identified the cysteine residue 285 in the ligand binding site of PPARγ as the site of covalent modification (33). Using mass spectrometry we indeed identified direct binding of compound **1a** to the highly reactive cysteine residue 368 in Keap1, suggesting that compound **1** and its analogues interact in a similar manner with Keap1 cysteine residues and thus activate Nrf2.

A remarkable feature of these new Nrf2 activators is the low toxicity for cultured keratinocytes, and both components were superior in this respect to SFN and tBHQ. Consistent with this finding, the capacity of compounds **1** and **1a** to induce the production of ROS was lower compared to tBHQ, but similar to SFN. In spite of this reduced toxicity, the UVB-protective effect of our new compounds was similar or even higher compared to SFN. These findings strongly suggest that compounds **1** and **1a** are interesting lead compounds for the development of drugs for skin protection under stress conditions. However, this will require establishment of an efficient *in vivo* delivery strategy. Thus, we only found a minor Nrf2-activating activity of compounds **1** and **1a** in preliminary experiments, while SFN induced Nrf2 target gene expression under the same conditions (data not shown). This is most likely due to inefficient penetration of compounds **1** and **1a** through the epidermal barrier. Therefore, it will be important in the future to improve the topical delivery, either through chemical modification of the compounds or through use of alternative delivery vehicles. In addition, the consequences of long-term *in vivo* application of the compounds need to be determined, in particular with regard to potential skin sensitization. In spite of these open issues, our results demonstrate that library screening combined with ligand-based virtual screening is a potent strategy to identify novel Nrf2 activators. The latter are starting points for hit-to-lead optimization aiming at the development of drugs that can be used for skin protection *in vivo*.

ACKNOWLEDGEMENTS

We thank Claudia Defila for providing the immortalized keratinocytes from Nrf2 knockout and wild-type mice, Dr. Yuet Wai Kan (University of California, San Francisco, CA) for Nrf2 knockout mice, Dr. Petra Boukamp, German Cancer Research Center Heidelberg for HaCaT keratinocytes, Drs. Walter Wahli and Liliane Michalik, University of Lausanne, for helpful suggestions with the PPAR experiments and Drs. Yong Chen and Martin Schulz, Institute of Pharmaceutical Sciences, ETH Zurich, for helpful advice with the library screen.

This work was supported by the Swiss National Science Foundation (grants 310030_132884/1 and CRSI33_130576/1 to S.W), the Promedica Foundation, Chur, Switzerland (to S.W.) and the CE.R.I.E.S. award (to S.W.). F.L. was supported by a PhD fellowship from the "Studienstiftung des Deutschen Volkes" and M.S. was supported by an EMBO postdoctoral fellowship.

REFERENCES

1. Bickers, D. R., and Athar, M. (2006) Oxidative stress in the pathogenesis of skin disease. *J Invest Dermatol.* **126**, 2565-2575
2. Schafer, M., and Werner, S. (2008) Oxidative stress in normal and impaired wound repair. *Pharmacol. Res.* **58**, 165-171
3. Braun, S., Hanselmann, C., Gassmann, M. G., auf dem Keller, U., Born-Berclaz, C., Chan, K., Kan, Y. W., and Werner, S. (2002) Nrf2 transcription factor, a novel target of keratinocyte growth factor action which regulates gene expression and inflammation in the healing skin wound. *Mol. Cell Biol.* **22**, 5492-5505
4. Motohashi, H., O'Connor, T., Katsuoka, F., Engel, J. D., and Yamamoto, M. (2002) Integration and diversity of the regulatory network composed of Maf and CNC families of transcription factors. *Gene* **294**, 1-12
5. Sykiotis, G. P., and Bohmann, D. (2010) Stress-activated cap'n'collar transcription factors in aging and human disease. *Sci. Signal.* **3**, re3
6. Holland, R., and Fishbein, J. C. (2010) Chemistry of the cysteine sensors in Kelch-like ECH-associated protein 1. *Antioxid. Redox Signal.* **13**, 1749-1761
7. Tong, K. I., Kobayashi, A., Katsuoka, F., and Yamamoto, M. (2006) Two-site substrate recognition model for the Keap1-Nrf2 system: a hinge and latch mechanism. *Biol. Chem.* **387**, 1311-1320
8. Itoh, K., Tong, K. I., and Yamamoto, M. (2004) Molecular mechanism activating Nrf2-Keap1 pathway in regulation of adaptive response to electrophiles. *Free Radic. Biol. Med.* **36**, 1208-1213
9. Durchdewald, M., Beyer, T. A., Johnson, D. A., Johnson, J. A., Werner, S., and auf dem Keller, U. (2007) Electrophilic chemicals but not UV irradiation or reactive oxygen species activate Nrf2 in keratinocytes in vitro and in vivo. *J. Invest. Dermatol.* **127**, 646-653
10. Rushmore, T. H., Morton, M. R., and Pickett, C. B. (1991) The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. *J. Biol. Chem.* **266**, 11632-11639
11. Yu, X., and Kensler, T. (2005) Nrf2 as a target for cancer chemoprevention. *Mutat. Res.* **591**, 93-102
12. auf dem Keller, U., Huber, M., Beyer, T. A., Kumin, A., Siemes, C., Braun, S., Bugnon, P., Mitropoulos, V., Johnson, D. A., Johnson, J. A., Hohl, D., and Werner, S. (2006) Nrf transcription factors in keratinocytes are essential for skin tumor prevention but not for wound healing. *Mol. Cell Biol.* **26**, 3773-3784
13. Xu, C., Huang, M. T., Shen, G., Yuan, X., Lin, W., Khor, T. O., Conney, A. H., and Kong, A. N. (2006) Inhibition of 7,12-dimethylbenz(a)anthracene-induced skin tumorigenesis in C57BL/6 mice by sulforaphane is mediated by nuclear factor E2-related factor 2. *Cancer Res.* **66**, 8293-8296
14. Kensler, T. W., Chen, J. G., Egner, P. A., Fahey, J. W., Jacobson, L. P., Stephenson, K. K., Ye, L., Coady, J. L., Wang, J. B., Wu, Y., Sun, Y., Zhang, Q. N., Zhang, B. C., Zhu, Y. R., Qian, G. S., Carmella, S. G., Hecht, S. S., Benning, L., Gange, S. J., Groopman, J. D., and Talalay, P. (2005) Effects of glucosinolate-rich broccoli sprouts on urinary levels of aflatoxin-DNA adducts and phenanthrene tetraols in a randomized clinical trial in He Zuo township, Qidong, People's Republic of China. *Cancer Epidemiol. Biomarkers Prev.* **14**, 2605-2613
15. O'Dwyer, P. J., Szarka, C. E., Yao, K. S., Halbherr, T. C., Pfeiffer, G. R., Green, F., Gallo, J. M., Brennan, J., Frucht, H., Goosenberg, E. B., Hamilton, T. C., Litwin, S., Balshem, A. M., Engstrom, P. F., and Clapper, M. L. (1996) Modulation of gene expression in subjects at risk for colorectal cancer by the chemopreventive dithiolethione oltipraz. *J. Clin. Invest.* **98**, 1210-1217
16. Schafer, M., Dutsch, S., auf dem Keller, U., Navid, F., Schwarz, A., Johnson, D. A., Johnson, J. A., and Werner, S. (2010) Nrf2 establishes a glutathione-mediated gradient of UVB cytoprotection in the epidermis. *Genes Dev.* **24**, 1045-1058
17. Dinkova-Kostova, A. T., Holtzclaw, W. D., Cole, R. N., Itoh, K., Wakabayashi, N., Katoh, Y., Yamamoto, M., and Talalay, P. (2002) Direct evidence that sulfhydryl groups of Keap1 are the

sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proc. Natl. Acad. Sci. U S A* **99**, 11908-11913
18. Dinkova-Kostova, A. T., Jenkins, S. N., Fahey, J. W., Ye, L., Wehage, S. L., Liby, K. T., Stephenson, K. K., Wade, K. L., and Talalay, P. (2006) Protection against UV-light-induced skin carcinogenesis in SKH-1 high-risk mice by sulforaphane-containing broccoli sprout extracts. *Cancer Lett.* **240**, 243-252
19. Juge, N., Mithen, R. F., and Traka, M. (2007) Molecular basis for chemoprevention by sulforaphane: a comprehensive review. *Cell. Mol. Life Sci.* **64**, 1105-1127
20. Kerns, M., DePianto, D., Yamamoto, M., and Coulombe, P. A. (2010) Differential modulation of keratin expression by sulforaphane occurs via Nrf2-dependent and -independent pathways in skin epithelia. *Mol. Biol. Cell* **21**, 4068-4075
21. Schafer, M., Farwanah, H., Willrodt, A. H., Huebner, A. J., Sandhoff, K., Roop, D., Hohl, D., Bloch, W., and Werner, S. (2012) Nrf2 links epidermal barrier function with antioxidant defense. *EMBO Mol. Med.* **4**, 364-379
22. Emter, R., Ellis, G., and Natsch, A. (2010) Performance of a novel keratinocyte-based reporter cell line to screen skin sensitizers in vitro. *Toxicol. Appl. Pharmacol.* **245**, 281-290
23. Chan, K., Lu, R., Chang, J. C., and Kan, Y. W. (1996) NRF2, a member of the NFE2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development. *Proc. Natl. Acad. Sci. U S A* **93**, 13943-13948
24. Eggler, A. L., Liu, G., Pezzuto, J. M., van Breemen, R. B., and Mesecar, A. D. (2005) Modifying specific cysteines of the electrophile-sensing human Keap1 protein is insufficient to disrupt binding to the Nrf2 domain Neh2. *Proc. Natl. Acad. Sci. U S A* **102**, 10070-10075
25. Jeong, W. S., Keum, Y. S., Chen, C., Jain, M. R., Shen, G., Kim, J. H., Li, W., and Kong, A. N. (2005) Differential expression and stability of endogenous nuclear factor E2-related factor 2 (Nrf2) by natural chemopreventive compounds in HepG2 human hepatoma cells. *J. Biochem. Mol. Biol.* **38**, 167-176
26. Tanigawa, S., Fujii, M., and Hou, D. X. (2007) Action of Nrf2 and Keap1 in ARE-mediated NQO1 expression by quercetin. *Free Radic. Biol. Med.* **42**, 1690-1703
27. Dinkova-Kostova, A. T., Abeygunawardana, C., and Talalay, P. (1998) Chemoprotective properties of phenylpropenoids, bis(benzylidene)cycloalkanones, and related Michael reaction acceptors: correlation of potencies as phase 2 enzyme inducers and radical scavengers. *J. Med. Chem.* **41**, 5287-5296
28. Nguyen, T., Huang, H. C., and Pickett, C. B. (2000) Transcriptional regulation of the antioxidant response element. Activation by Nrf2 and repression by MafK. *J. Biol. Chem.* **275**, 15466-15473
29. Kensler, T. W., Wakabayashi, N., and Biswal, S. (2007) Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu. Rev. Pharmacol. Toxicol.* **47**, 89-116
30. Tanrikulu, Y., Nietert, M., Scheffer, U., Proschak, E., Grabowski, K., Schneider, P., Weidlich, M., Karas, M., Gobel, M., and Schneider, G. (2007) Scaffold hopping by "fuzzy" pharmacophores and its application to RNA targets. *Chembiochem.* **8**, 1932-1936
31. Reisen, F., Weisel, M., Kriegl, J. M., and Schneider, G. (2010) Self-organizing fuzzy graphs for structure-based comparison of protein pockets. *J. Proteome Res.* **9**, 6498-6510
32. Geppert, T., Reisen, F., Pillong, M., Hahnke, V., Tanrikulu, Y., Koch, C. P., Perna, A. M., Perez, T. B., Schneider, P., and Schneider, G. (2012) Virtual screening for compounds that mimic protein-protein interface epitopes. *J. Comput. Chem.* **33**, 573-579
33. Leesnitzer, L. M., Parks, D. J., Bledsoe, R. K., Cobb, J. E., Collins, J. L., Consler, T. G., Davis, R. G., Hull-Ryde, E. A., Lenhard, J. M., Patel, L., Plunket, K. D., Shenk, J. L., Stimmel, J. B., Therapontos, C., Willson, T. M., and Blanchard, S. G. (2002) Functional consequences of cysteine modification in the ligand binding sites of peroxisome proliferator activated receptors by GW9662. *Biochemistry* **41**, 6640-6650

34. Mastrofrancesco, A., Ottaviani, M., Aspite, N., Cardinali, G., Izzo, E., Graupe, K., Zouboulis, C. C., Camera, E., and Picardo, M. (2010) Azelaic acid modulates the inflammatory response in normal human keratinocytes through PPARgamma activation. *Exp. Dermatol.* **19**, 813-820
35. Lee, G., Elwood, F., McNally, J., Weiszmann, J., Lindstrom, M., Amaral, K., Nakamura, M., Miao, S., Cao, P., Learned, R. M., Chen, J. L., and Li, Y. (2002) T0070907, a selective ligand for peroxisome proliferator-activated receptor gamma, functions as an antagonist of biochemical and cellular activities. *J. Biol. Chem.* **277**, 19649-19657
36. Wright, H. M., Clish, C. B., Mikami, T., Hauser, S., Yanagi, K., Hiramatsu, R., Serhan, C. N., and Spiegelman, B. M. (2000) A synthetic antagonist for the peroxisome proliferator-activated receptor gamma inhibits adipocyte differentiation. *J. Biol. Chem.* **275**, 1873-1877
37. Lehmann, J. M., Moore, L. B., Smith-Oliver, T. A., Wilkison, W. O., Willson, T. M., and Kliewer, S. A. (1995) An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). *J. Biol. Chem.* **270**, 12953-12956
38. Hayes, J. D., McMahon, M., Chowdhry, S., and Dinkova-Kostova, A. T. (2010) Cancer chemopreventive mechanisms mediated through the Keap1-Nrf2 pathway. *Antioxid. Redox. Signal.* **13,** 1713-48
39. Hong, F., Freeman, M. L., and Liebler, D. C. (2005) Identification of sensor cysteines in human Keap1 modified by the cancer chemopreventive agent sulforaphane. *Chem. Res. Toxicol.* **18**, 1917-1926
40. Talalay, P., Fahey, J. W., Healy, Z. R., Wehage, S. L., Benedict, A. L., Min, C., and Dinkova-Kostova, A. T. (2007) Sulforaphane mobilizes cellular defenses that protect skin against damage by UV radiation. *Proc. Natl. Acad. Sci. U S A* **104**, 17500-17505
41. Shapiro, T. A., Fahey, J. W., Dinkova-Kostova, A. T., Holtzclaw, W. D., Stephenson, K. K., Wade, K. L., Ye, L., and Talalay, P. (2006) Safety, tolerance, and metabolism of broccoli sprout glucosinolates and isothiocyanates: a clinical phase I study. *Nutr. Cancer* **55**, 53-62
42. Zhu, M., Baek, H., Liu, R., Song, A., Lam, K., and Lau, D. (2009) LAS0811: from combinatorial chemistry to activation of antioxidant response element. *J. Biomed. Biotechnol.* **2009**, 420194
43. Hur, W., Sun, Z., Jiang, T., Mason, D. E., Peters, E. C., Zhang, D. D., Luesch, H., Schultz, P. G., and Gray, N. S. (2010) A small-molecule inducer of the antioxidant response element. *Chem. Biol.* **17**, 537-547
44. Smirnova, N. A., Haskew-Layton, R. E., Basso, M., Hushpulian, D. M., Payappilly, J. B., Speer, R. E., Ahn, Y. H., Rakhman, I., Cole, P. A., Pinto, J. T., Ratan, R. R., and Gazaryan, I. G. (2011) Development of Neh2-luciferase reporter and its application for high throughput screening and real-time monitoring of Nrf2 activators. *Chem. Biol.* **18**, 752-765
45. Schneider, G. (2010) Virtual screening: an endless staircase? *Nat Rev Drug Discov* 9, 273-276
46. Dinkova-Kostova, A. T., Holtzclaw, W. D., and Kensler, T. W. (2005) The role of Keap1 in cellular protective responses. *Chem. Res. Toxicol.* **18**, 1779-1791
47. Kerns, M. L., DePianto, D., Dinkova-Kostova, A. T., Talalay, P., and Coulombe, P. A. (2007) Reprogramming of keratin biosynthesis by sulforaphane restores skin integrity in epidermolysis bullosa simplex. *Proc. Natl. Acad. Sci. U S A* **104**, 14460-14465
48. Wu, J., Xia, C., Meier, J., Li, S., Hu, X., and Lala, D. S. (2002) The hypolipidemic natural product guggulsterone acts as an antagonist of the bile acid receptor. *Mol Endocrinol* 16, 1590-1597
49. Shishodia, S., and Aggarwal, B. B. (2004) Guggulsterone inhibits NF-kappaB and IkappaBalpha kinase activation, suppresses expression of anti-apoptotic gene products, and enhances apoptosis. *J. Biol. Chem.* **279**, 47148-47158
50. Medana, C., Ermondi, G., Fruttero, R., Di Stilo, A., Ferretti, C., and Gasco, A. (1994) Furoxans as nitric oxide donors. 4-Phenyl-3-furoxancarbonitrile: thiol-mediated nitric oxide release and biological evaluation. *J. Med. Chem.* **37**, 4412-4416
51. Li, C. Q., Kim, M. Y., Godoy, L. C., Thiantanawat, A., Trudel, L. J., and Wogan, G. N. (2009) Nitric oxide activation of Keap1/Nrf2 signaling in human colon carcinoma cells. *Proc. Natl. Acad. Sci. U S A* **106**, 14547-14551

52. McMahon, M., Lamont, D. J., Beattie, K. A., and Hayes, J. D. (2010) Keap1 perceives stress via three sensors for the endogenous signaling molecules nitric oxide, zinc, and alkenals. *Proc. Natl. Acad. Sci. U S A* **107**, 18838-18843
53. Schneider, G., Neidhart, W., Giller, T., and Schmid, G. (1999) "Scaffold-Hopping" by Topological Pharmacophore Search: A Contribution to Virtual Screening. *Angew. Chem. Int. Ed. Engl.* **38**, 2894-2896

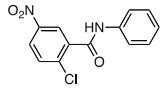
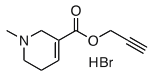
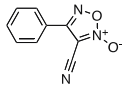
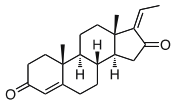
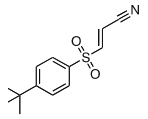
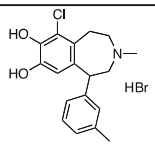
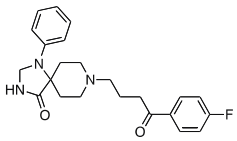
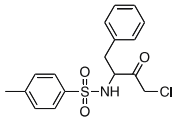
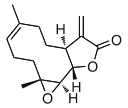
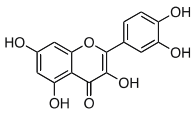
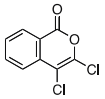
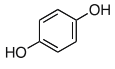
No.	Compound	Structure	Main target according to LOPAC library	Action	Described as Nrf2 activator	Discovered in x screening experiments
1	2-Chloro-5-nitro-N-phenyl-benzamide (GW9662)		PPARγ	Inhibitor	no	3x
2	Arecaidine propargyl ester hydrobromide		M2 receptors	Agonist	no	3x
3	4-Phenyl-3-furoxan-carbonitrile			NO Donor	no	3x
4	(Z)-Gugglesterone		FXR	Antagonist	no	3x
5	Bay 11-7085		IκBα	Inhibitor	no	3x
6	SKF 83959 hydrobromide		D1	Agonist	no	2x
7	Spiperone hydrochloride		D2	Antagonist	no	2x
8	Tosyl-L-phenyl-alanine chloromethyl ketone		Chymotrypsin α	Inhibitor	no	2x
9	Iodoacetamide		Cysteine peptidases	Inhibitor	yes[23]	3x
10	Parthenolide		IκBα	Inhibitor	yes[24]	2x
11	Quercetin dihydrate		PDE	Inhibitor	yes[25]	2x
12	3,4-Dichloroiso-coumarin		Serine protease	Inhibitor	shown for coumarin[26]	2x
13	Hydroquinone		Arachidonate 12-lipoxygenase	Inhibitor	shown for tBHQ[27]	2x

Table 1. List of compounds that activated the luciferase reporter gene in at least two screening experiments. Additional information on the structure, targets and mechanisms of action of these compounds are provided in the description of the LOPAC library (Sigma) (PPARγ - peroxisome proliferator-activated receptor gamma, M2 receptor - muscarinic acetylcholine receptor M2, NO - nitric oxide, FXR - farnesoid X receptor, IκBα - nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor α, D1/2 - dopamine receptor D1/D2, PDE - phosphodiesterase).

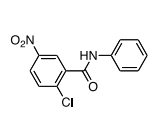
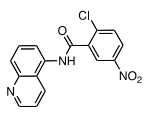
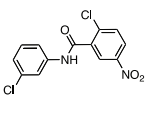
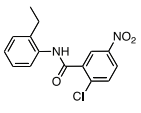
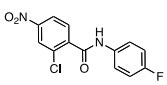
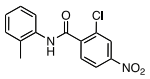
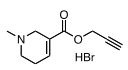
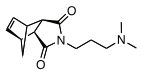
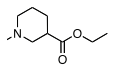
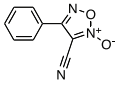
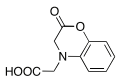
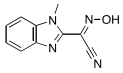
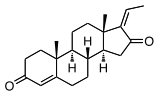
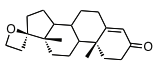
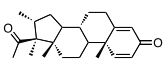
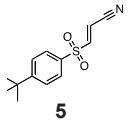
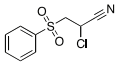
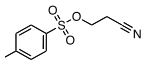
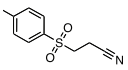
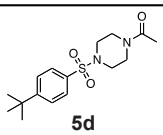
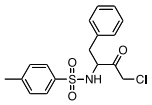
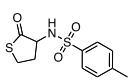
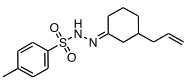
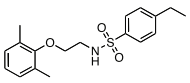
Hit compounds	Predicted Nrf2 activators				
 1	 1a	 1b	 1c	 1d	 1e
 2	 2a	 2b	 2c	 2d	
 3	 3a	 3b			
 4	 4a	 4b			
 5	 5a	 5b	 5c	 5d	
 8	 8a	 8b	 8c		

Table 2. List of compounds identified in the virtual screen. Compounds, which increased reporter gene expression, are highlighted.

FIGURE LEGENDS

Figure 1. Dose-dependent activation of luciferase activity by eight hit compounds. (A, B) HaCaT cells were transfected with the pGL3-promoter vector or with the pGL3-8xARE-mGclm reporter plasmid and treated for 24 h with different concentrations of the hit compounds as indicated or with 50 µM tBHQ or 5 µM SFN. Luciferase activities were determined in triplicates and normalized to luciferase activities seen in DMSO-treated cells. Bars represent mean of three independent experiments ±SD.

Figure 2. Induction of Nrf2 target gene expression by treatment with the identified hit compounds. (A-C) HaCaT cells were treated for 8 h, 24 h or 48 h with tBHQ (50 µM), SFN (5 µM), or the different hit compounds. Compounds **3**, **5**, **6**, **7** and **8** were used at a concentration of 10 µM, compounds **1** and **4** at a concentration of 25 µM and compound **2** at a concentration of 50 µM. RNA was isolated from the treated cells and analyzed for expression of the Nrf2 target genes *GCLC, GCLM* and *NQO1* by qRT-PCR. *RPL27* was used for normalization. The expression level in DMSO-treated HaCaT cells was set to 1. Values represent mean of three independent experiments ±SD.

Figure 3. Hit compounds activate Nrf2 target genes in immortalized keratinocytes from wild-type but not from Nrf2 knockout mice (A-C) Spontaneously immortalized murine keratinocytes from wild-type or Nrf2 knockout mice were treated for 8 h with tBHQ (50 µM), SFN (5 µM), or the hit compounds using the concentrations listed in the legend to Fig.2. RNA samples were analyzed for expression of the Nrf2 target genes *Gclm*, *Gclc* and *Nqo1* by qRT-PCR. *Rps29* was used for normalization. The expression levels in DMSO-treated keratinocytes of each genotype were set to 1. Bars represent mean of three independent experiments ±SD.

Figure 4. Hit compounds and some of their analogues activate *Gclc/GCLC* in immortalized keratinocytes and in primary human foreskin keratinocytes. (A) Spontaneously immortalized murine keratinocytes from wild-type and Nrf2 knockout mice were treated for 8 h with tBHQ (50 µM), SFN (5 µM), compounds **1** (25 µM), **1a** (25 µM), **1b** (25 µM), **2** (50 µM), **5** (10 µM) and **5a** (10 µM). RNA samples were analyzed for expression of the Nrf2 target gene *Gclc* by qRT-PCR. *Rps29* was used for normalization. The expression levels in DMSO-treated keratinocytes of each genotype were set to 1. **(B)** Primary human foreskin keratinocytes were treated for 24 h with tBHQ (50 µM), SFN (5 µM), compounds **1** (50 µM), **1a** (50 µM), **1b** (50 µM), **2** (50 µM), **5** (5 µM) and **5a** (5 µM). RNA samples were analyzed for expression of the Nrf2 target gene *GCLC* by qRT-PCR. *RPL27* was used for normalization. The expression levels in DMSO-treated keratinocytes were set to 1. Bars represent mean of three independent experiments ±SD.

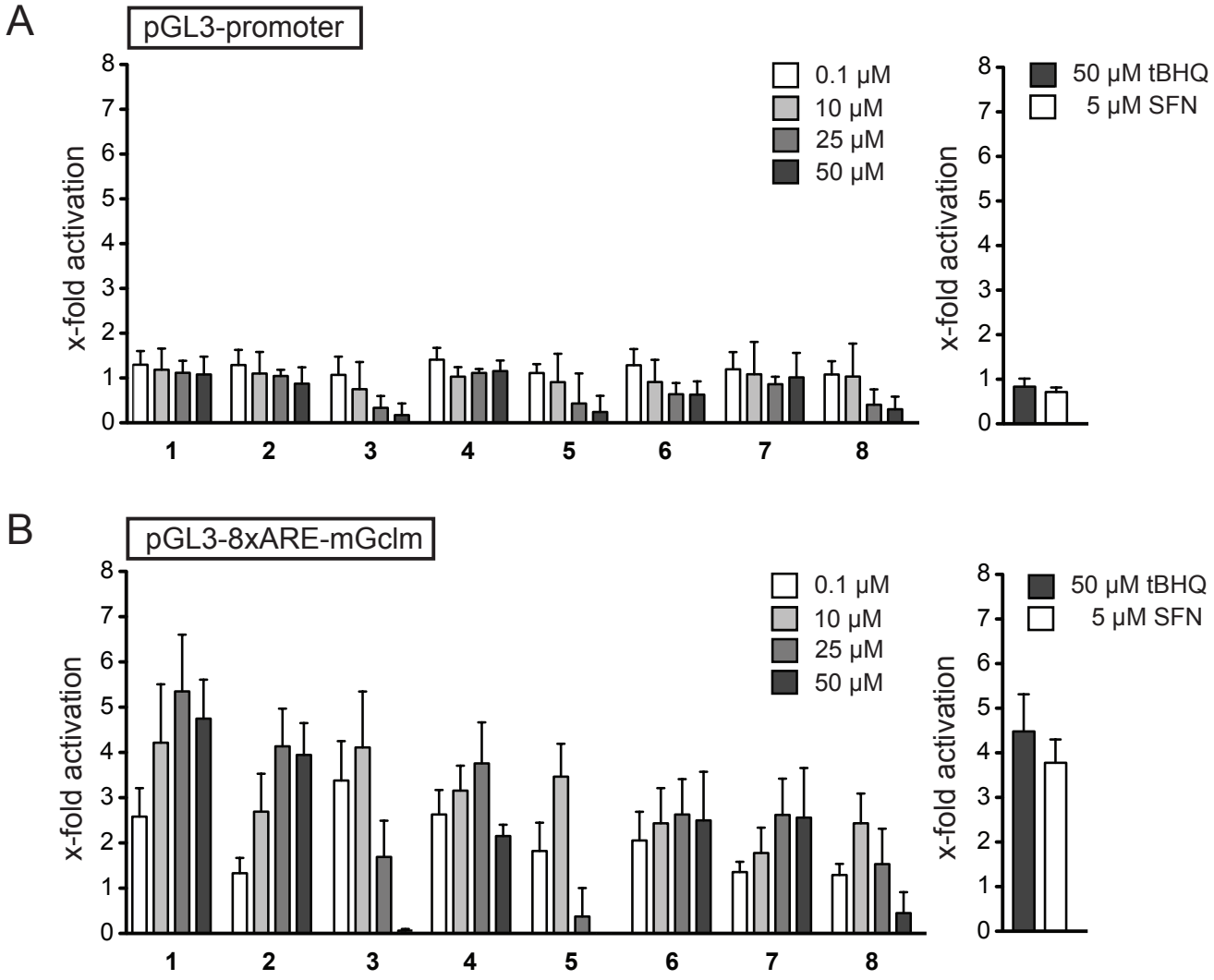
Figure 5. Upregulation of Nrf2 target genes by compounds 1 and 1a is not dependent on PPARγ inhibition. (A, B) Primary human foreskin keratinocytes were treated for 24 h with tBHQ (50 µM), SFN (5 µM), compounds **1** (50 µM), **1a** (50 µM), BADGE (10 µM), T0070907 (10 µM) or rosiglitazone (10 µM). RNA samples were analyzed for expression of *GCLC* and *NQO1* by qRT-PCR. *RPL27* was used for normalization. The expression level in DMSO-treated keratinocytes was set to 1. Bars represent mean of three independent experiments ±SD.

Figure 6. Reduced toxicity of compounds 1 and 1a compared to tBHQ and sulforaphane. (A, B) Primary human foreskin keratinocytes were treated with tBHQ, SFN, compound **1** or compound **1a** at different concentrations or with the solvent DMSO. Cell viability was determined after 24 h incubation using MTT assay **(A)** or by measurement of LDH activity in the cell supernatant **(B)**. **(C)** Cells were treated with the solvent DMSO or with 5 µM SFN, 25 µM tBHQ, 50 µM compound **1** or 50 µM compound **1a** and analyzed at different time points for the presence of intracellular ROS by DCF fluorescence measurement. Values from triplicate determinations are shown. Results shown in **(A-C)** were repeated in an independent experiment with a different batch of cells.

Figure 7. Compounds 1 and 1a protect keratinocytes from UVB toxicity. (A, B) Primary human foreskin keratinocytes were plated on 12-well plates and treated with DMSO, SFN (5 µM), compound **1** (50 µM) or compound **1a** (50 µM) for 1 h, 4 h or 20 h as indicated. After replacing the medium with fresh medium, cells were irradiated with a single dose of UVB (50 mJ/cm^2). Cytotoxicity was determined by LDH release **(A)**. Bars represent mean of three independent experiments ±SD. Two-way ANOVA analysis showed significantly reduced LDH-release in cells pretreated with compounds **1** or **1a** for 4 h or 20 h when compared to vehicle control. Alternatively, cell viability was determined using MTT assay and analyzed in triplicate dishes **(B)**. Results of a representative experiment out of four independent assays are shown.

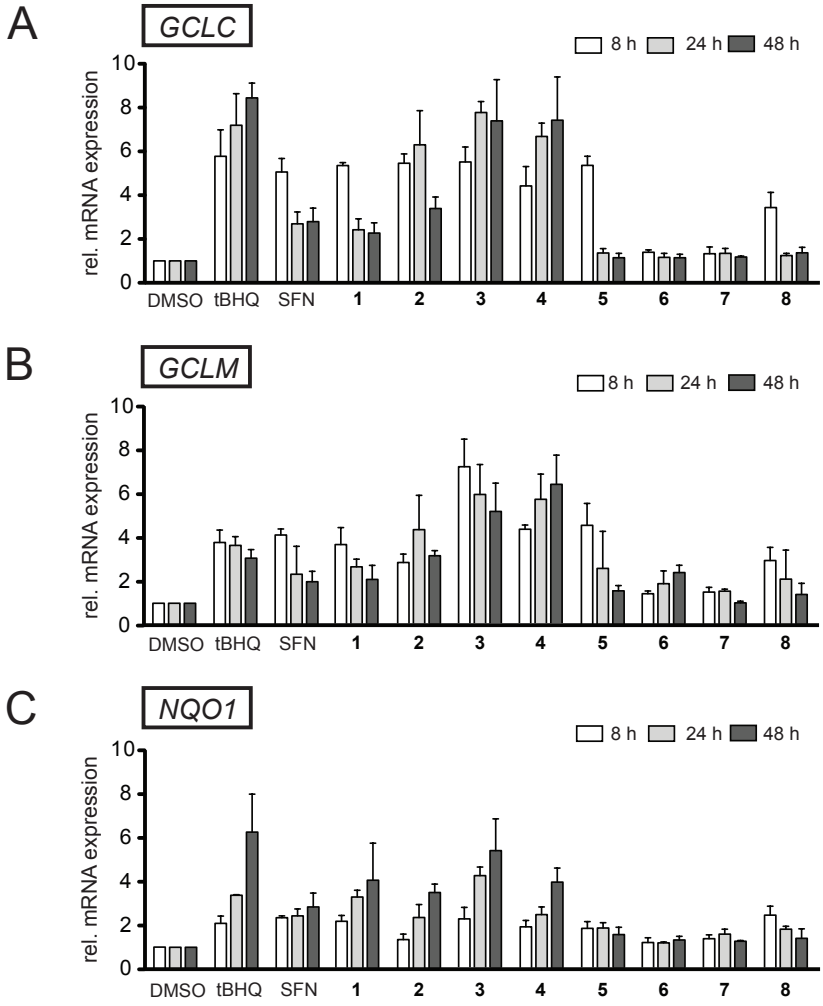
Lieder et al.

Figure 1



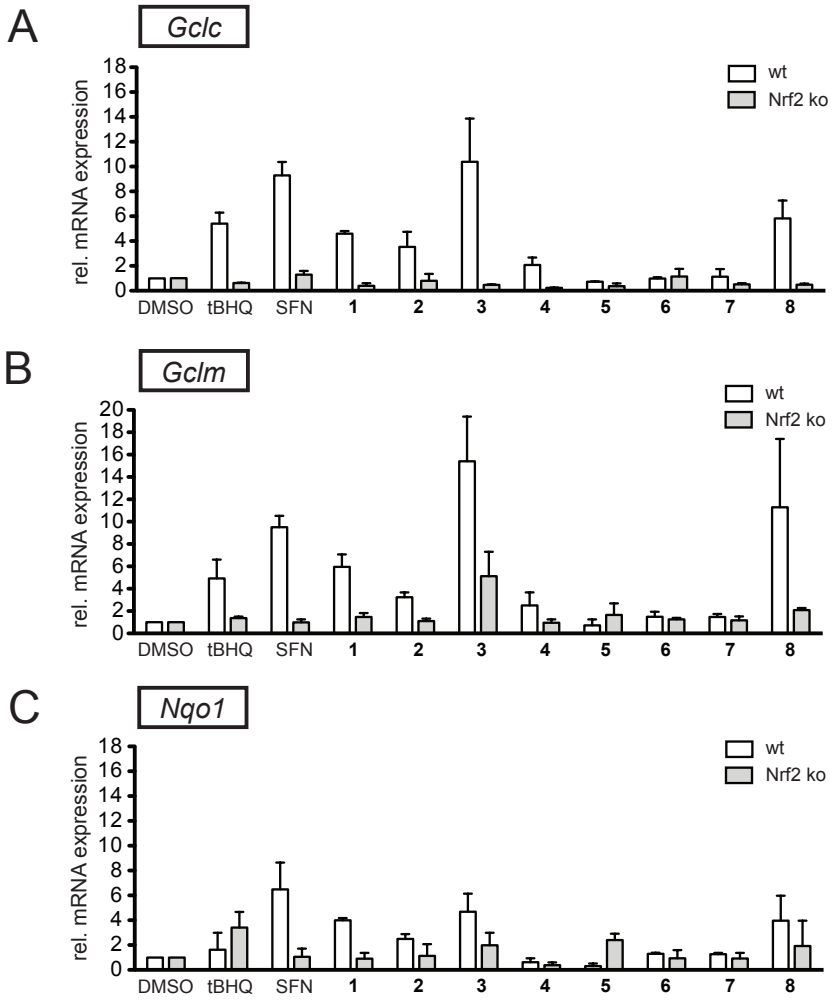
Lieder et al.

Figure 2



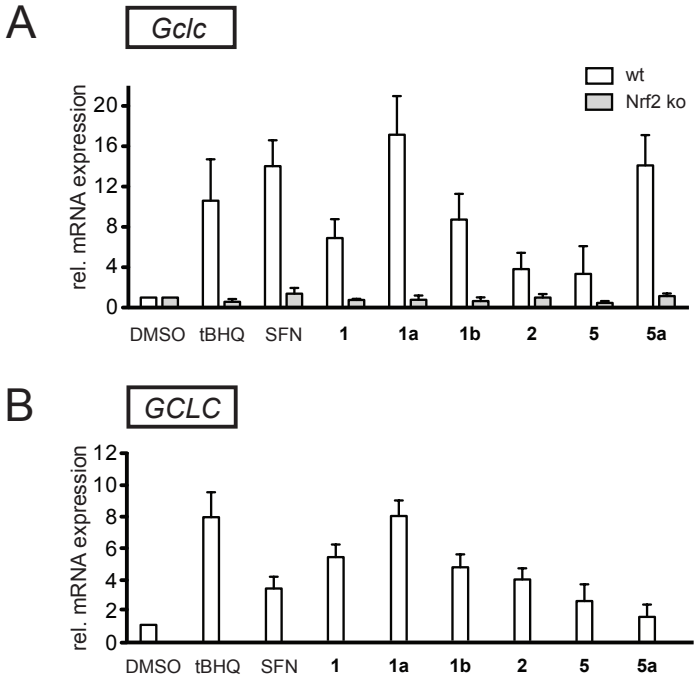
Lieder et al.

Figure 3



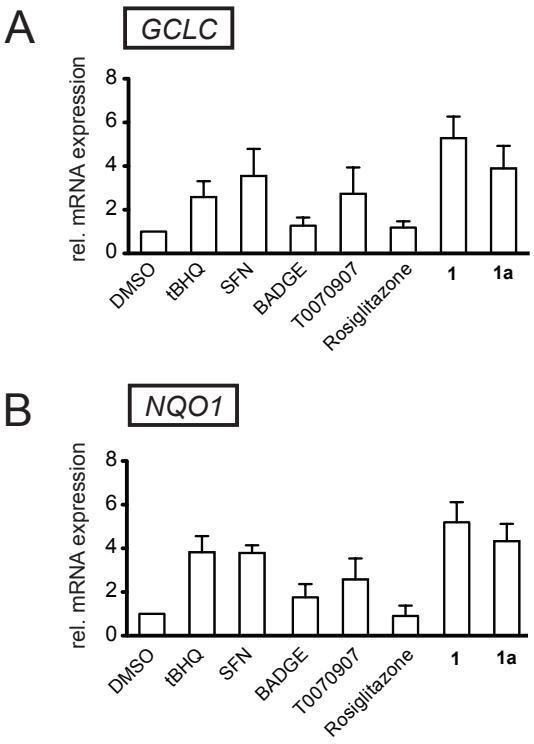
Lieder et al.

Figure 4



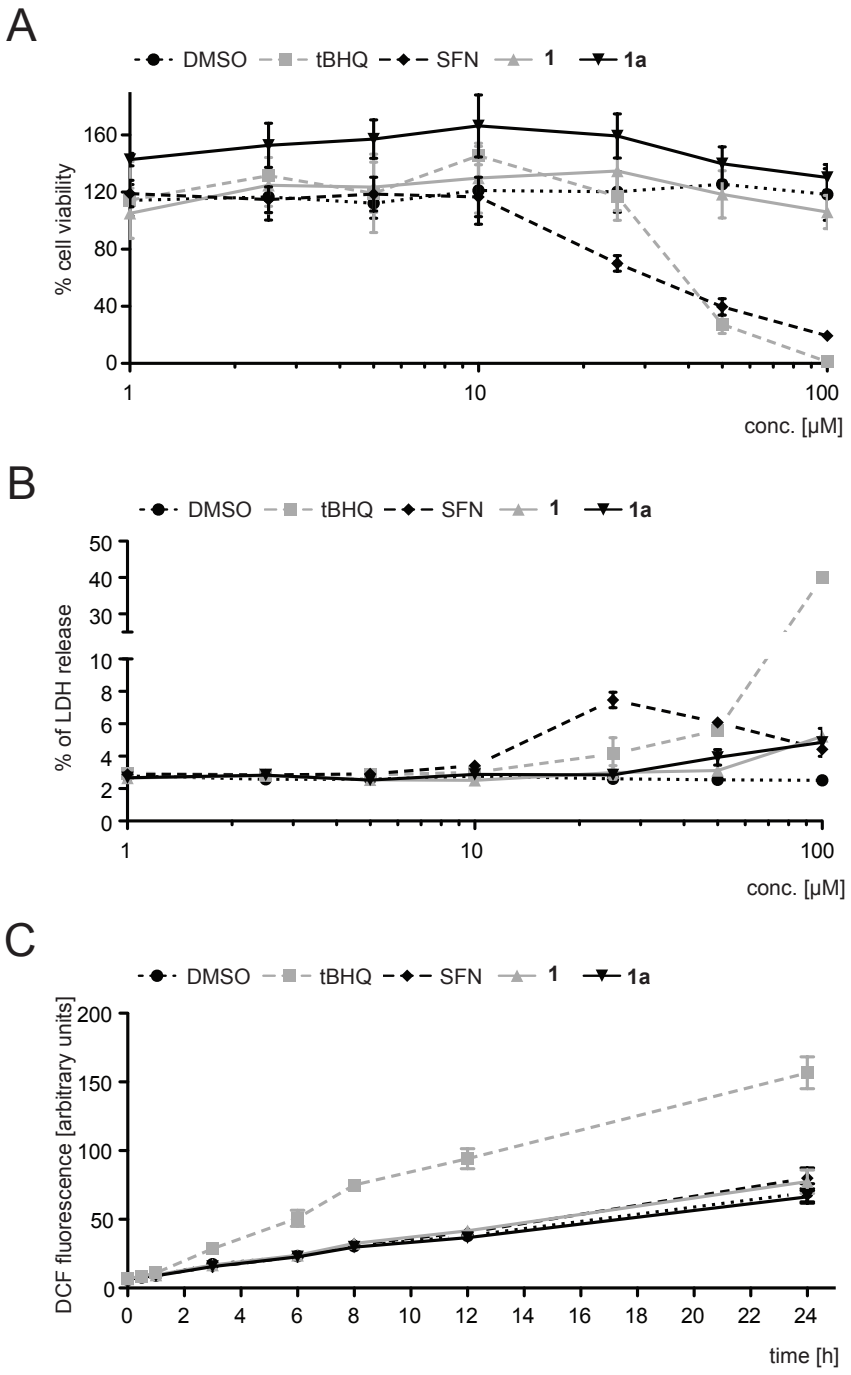
Lieder et al.

Figure 5



Lieder et al.

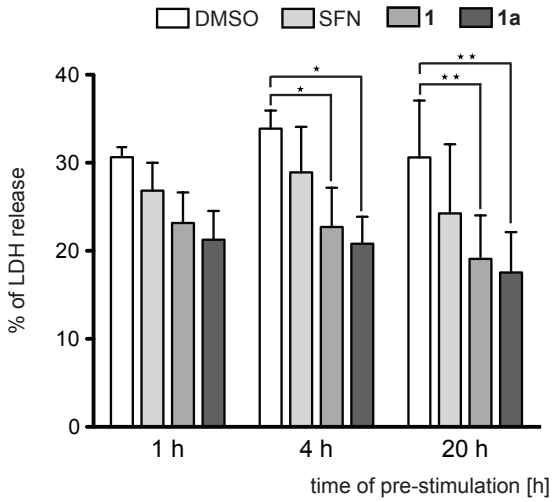
Figure 6



Lieder et al.

Figure 7

A



B